JOURNAL OF VIROLOGY, Apr. 1976, p. 117-123
Vol. 18, No. 1

Printed in U.S.A.

Recombination Between Temperature-Sensitive and Deletion Mutants of Reovirus

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Received for publication 22 September 1975

In standard pairwise crosses there was no detectable recombination between defective reovirus lacking the largest genomic segment and prototypes of the seven known classes of *ts* mutants. However, in such crosses between R_2A (201) and the various prototypes frequencies of ts^+ recombinants between 2.6 and 6.1% were observed, as others have found (Fields, 1971; Fields and Joklik, 1969). An infectious center assay was devised to measure recombination in this system, and it was found that all mixedly infected cells gave rise to ts^+ recombinants in crosses between prototype *ts* mutants, but no recombination was detectable when the defective virus was crossed with three different *ts* mutants. The *ts* mutation of mutant R_2A (201) was efficiently rescued when crossed with UV-inactivated wild-type virus but not when crossed with UV-inactivated defective virus. It is concluded from these various experiments that if there is any recombination between these defective reovirions and any known class of *ts* mutants it is too low to be measured by methods presently available. The kinetics of recombination were measured in cells mixedly infected with R_2A (201) and R_2B (352) mutants. At the earliest time progeny virus could be found in the cells the frequency of ts^+ recombinants was 4.5%, and this frequency remained unchanged despite a subsequent 1,000-fold increase in progeny virus.

Temperature-sensitive (*ts*) mutants of reovirus type 3 have been classified by their recombination frequencies in pairwise crosses (2, 3). In such mixed infections recombination was found to be rather high, of the order of 3 to 8% ts^+ recombinants, or virtually undetectable. The known mutants have thus been placed in seven classes, A through G, each class thought to represent mutations in a corresponding one of the ten double-stranded RNA (dsRNA) segments of the genome. Since there is no evidence as yet of genetic linkage between the mutant classes, it has been proposed and generally accepted that recombination with this virus occurs through the reassortment of whole genomic segments.

One of the major problems with the reovirus system is then to allocate each class of *ts* mutants to its specific segment, and to this end we have been attempting to obtain deletion mutants of the virus to use in genetic tests. Recently we described the isolation of a defective virion lacking the largest (L_1) segment of the genome (14). Complementation tests between this defective virion and the various known classes of *ts* mutants indicated that the class C mutant was the only one that neither complemented nor was complemented by the defective virion (13). The function in which the C mutation occurred was therefore assigned to the L_1 segment of the viral genome. In an attempt to get further evidence for this assignment we have determined the recombination frequencies in crosses between the defective virions and various *ts* mutants. These experiments are the subject of the present paper and have led to the unexpected result that there is no detectable recombination between the defectives and any known class of *ts* mutants.

MATERIALS AND METHODS

Cells and virus. L cells were grown in suspension in complete medium, which consists of Eagle minimum essential medium (MEM) supplemented with 5% fetal calf serum.

Buffers, chemicals, labeled precursors, the conditions for growth, and purification of standard (infectious) virus and defective virions have been described (14).

The wild-type strain of reovirus serotype 3, R_2^3, was used. Since there is no confusion with other serotypes of the virus in this paper the wild-type strain will be designated R_2. The following *ts* mutants of R_2 virus isolated by Fields et al. (2, 3) have been used: R_2A (201), R_2B (352), R_2C (447), R_2D (357), R_2D (585), R_2E (320), R_2F (556), and R_2G (453). These mutants were kindly supplied by B. N. Fields. The nomenclature used for the mutants has been described (14), with use of the letters A, B, C,

etc., referring to the mutant group defined by genetic recombination in pairwise crosses (2, 3) and the number in parentheses referring to the specific mutant. The deletion mutant R_1d (L_1) has also been used. This is a defective virion lacking the L_1 segment of the genome whose isolation has been described (14). Permissive temperature for the plaque assay (9) was 31 C, and the nonpermissive temperature was 39 C. For the present work all virus strains were freshly cloned. The wild-type strain and mutants as received from B. N. Fields were plaque-titered at 31 C, and an isolated plaque was picked. Each plaque was grown up into a large stock in L cells, and virus from the final lysates was then purified for use in the various experiments. The titers of each of these stocks at 31 C and 39 C have been reported (13). In all cases the efficiency of plating, 39 C/31 C, was less than 3×10^{-4}.

Standard recombination test. In this test the ability of any two mutants to give rise to ts^+ recombinants was determined by infecting a monolayer culture (approximately 5×10^6 cells in a screw-cap bottle) with the two mutants at an added multiplicity of infection (MOI) to be later specified. Similar cell cultures were infected with each mutant separately. After 1 h at room temperature to permit virus adsorption approximately 80% of the virus was adsorbed, the cultures were washed twice with 5 ml of MEM and then 5 ml of MEM containing 2% fetal calf serum was added, and the cultures were placed at 31 C. Viral growth was terminated 30 h after infection by freezing the cultures at −70 C. When required the cultures were thawed, frozen and thawed three additional times, sonicated gently for 2 min, and plaque-titered at 31 C and 39 C. In some experiments the complementation index between two mutants was determined. The procedure for this test was the same as just described except that the mixedly infected plates were incubated for 18 h at 39 C prior to titration at 31 C and 39 C, and the complementation index has been defined (13).

The recombination frequency, defined as the percentage of ts^+ recombinants in the yield of the cross between any two mutants X and Y was calculated according to the formula (2)

$$\{[(XY_{31})^{39} - (X_{31})^{39} - (Y_{31})^{39}]/(XY_{31})^{31}\} \times 100$$

where $(XY_{31})^{39}$ and $(XY_{31})^{31}$ are the titers of mixed yield grown at 31 C but assayed at 39 C and 31 C, respectively, and $(X_{31})^{39}$ and $(Y_{31})^{39}$ are the titers of single yields grown at 31 C and assayed at 39 C.

Recombination by infectious center assay. Confluent monolayers of L cells in 60-mm plastic dishes were coinfected with two *ts* mutants of reovirus at an MOI of 10 PFU/cell for each mutant or one *ts* mutant (MOI of 10 PFU/cell) and defective virions (200 particles/cell). Adsorption of virus was permitted for 1 h at room temperature, and the cells were then suspended by digestion for 5 min with 2 ml of a 0.05% trypsin solution in SPE buffer (140 mM NaCl, 3 mM KCl, 12 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.5 mM EDTA disodium-dihydrate, pH 7.4). One milliliter of heat-inactivated fetal calf serum was added to the suspension, and the cells were centrifuged at $850 \times g$ for 10 min and resuspended in MEM containing 2% fetal calf serum. A known number of these infected cells (100 to 130) in 0.5 ml of medium were mixed with 3×10^6 to 4×10^6 uninfected L cells from a suspension culture in MEM-2% fetal calf serum, added to a 60-mm plastic petri dish, and placed at the required temperature. After 3 h, when the cells had attached to the dish, standard agar overlay was added, and the incubation continued under the conditions to be specified later. Plaques were scored after staining the plates with 0.01% neutral red in phosphate-buffered saline solution. The usual procedure in infectious center assays is to add the infected cells to preformed monolayers of uninfected cells and after time for them to attach to overlay with agar. The minor but very effective modification of this procedure used here, i.e., mixing the infected cells with uninfected cells prior to plating, was introduced by C. Legaré, who has permitted us to describe it here prior to her own publication. In this system the modified technique gives consistently higher (10 to 15%) and more uniform plating efficiencies for infectious centers.

UV inactivation of virus. To inactivate virus for the marker rescue experiments samples of purified virus in phosphate-buffered saline were sonicated for two 30-s intervals. This treatment broke up clumps of virus which produced a marked inflexion in the UV inactivation curve at approximately 10^{-2} survival of virus. Samples of sonicated virus, 2 ml, were placed in 60-mm plastic dishes and irradiated with UV light from a pair of 15-W GEC germicidal lamps at a distance of 30 cm (energy flux at the plate position was 15 ergs/mm^2 per s) for 6 min. These conditions reduced the titer of R_2 virus to at least $10^{-2.5}$ survival with single-hit kinetics. Purified defective virions were treated in exactly the same way.

RESULTS

Recombination between ts mutants. As a test of our system for assessing recombination frequency a standard cross was carried out between the A and B mutants. At the same time the two mutants were crossed in the standard complementation test (14). The results are shown in Table 1. A frequency of 4.5% ts^+ recombinants was found, similar to that reported in previous work (2, 3). The complementation index was 5, which is in the range previously reported for these two mutants (2, 13).

Recombination between ts mutants and $R_1{}^3d$ (L_1) virions. We have already shown that defective virions, $R_1{}^3d$ (L_1), complement the known classes of *ts* mutants with the exception of the C and E mutants, and it was therefore of interest to determine whether recombination would occur between *ts* mutants and the defective virions. Table 2 shows the results of standard complementation and recombination tests between defectives and the A class mutant. The complementation index of 2 is consistent with previous results (13), but virtually no recombination occurred.

TABLE 1. *Virus yields after single and mixed infections with R_2A (201) and R_2B (352)*[a]

Input MOI (PFU/cell)		Incubation temp (C)	Yield (PFU/ml) assayed at	
R_2A (201)	R_2B (352)		31 C	39 C
10	0	31	7.0×10^7	3.0×10^4
		39	5.0×10^3	1.5×10^3
0	10	31	6.0×10^7	2.0×10^4
		39	3.0×10^3	1.2×10^3
10	10	31	2.0×10^8	9.0×10^6
		39	5.5×10^4	1.5×10^4

[a] Complementation index, $(5.5 \times 10^4 - 1.5 \times 10^4)/(3.0 \times 10^3 + 5.0 \times 10^3) = 5$. Recombination frequency, $100 \times [(9.0 \times 10^6 - 3.0 \times 10^4 - 2.0 \times 10^4)/(2.0 \times 10^8)] = 4.5\%$ ts^+.

TABLE 2. *Virus yield after single and mixed infections with R_1d (L_1) and R_2A (201)*[a]

Input MOI		Incubation temp (C)	Yield (PFU/ml) assayed at	
R_1d (L_1) particles/cell	R_2A (201) PFU/cell		31 C	39 C
200	0	31	4.0×10^2	2.0×10^2
		39	1.5×10^2	1.4×10^2
0	10	31	7.0×10^7	3.0×10^4
		39	5.0×10^3	1.5×10^3
200	10	31	1.0×10^8	5.0×10^4
		39	2.1×10^4	1.0×10^4

[a] Complementation level, $(2.1 \times 10^4 - 10^4)/(1.5 \times 10^2 + 5.0 \times 10^3) = 2$. Recombination frequency, $[(5.0 \times 10^4 - 2.0 \times 10^2 - 1.5 \times 10^3)/10^8] \times 100 = 0.05\%$ ts^+.

The lack of recombination was unexpected, since both mutant and defective viruses replicate readily in the coinfection at 31 C (13, 14). To determine whether the MOIs might have some influence on the results, the MOIs of both parental viruses were varied through a wide range in standard recombination tests. No significant recombination was found between the A mutant and defectives at any combination of MOIs (Table 3).

It was conceivable that the lack of recombination found in the tests of Tables 2 and 3 was attributable to some peculiarity of the A mutant and that other *ts* mutants would behave in a different fashion. Standard tests were therefore carried out between defective virions and the prototypes from each of the known classes of *ts* mutants. As a control the A mutant was also crossed against the various classes of mutants. The results are shown in Table 4.

TABLE 3. *Effect of variation in MOIs on recombination between R_2A (201) and R_1d (L_1)*

R_2A (201) (PFU/cell)	R_1d (L_1) (particle/cell)		
	20	200	2,000
	Recombination frequency (% ts^+)		
1	<0.02	0.02	<0.02
10	0.02	0.05	0.03
100	0.03	0.05	0.04

Whereas the A mutant gave recombination frequencies in the expected range (2, 3), there was no significant recombination between the defectives and any class of *ts* mutants.

Detection of recombination by infectious center assay. To determine whether or not recombination occurs in pairwise crosses of a number of mutants is a tedious job if the standard recombination test is used. In an effort to develop a simple and rapid method we have turned to an infectious center assay of cells coinfected by two mutants, since this had proven an effective means of detecting recombination in other systems (5). The details of the technique are given in Materials and Methods.

In principle a monolayer culture is coinfected with given multiplicities of two mutants, the infected cells are suspended with trypsin, and a known number is mixed with approximately 3×10^6 uninfected cells and placed in a petri dish. When the cells have adhered to the dish they are overlayed and placed at 31 C.

During an interval at 31 C virus will begin to multiply in the infected cells and recombination should occur. The plates are then shifted to 39 C and any ts^+ recombinants that have formed should develop into plaques. As controls, similarly infected plates are kept continuously at 39 C and at 31 C, the former as an estimate of the background of ts^+ virus in the parental populations, the latter to estimate the total number of infected cells plated.

Table 5 shows the results of an experiment of this sort, which was designed to find the length of time the infectious centers must be kept at 31 C prior to raising the temperature to 39 C. Cells were infected with either the A or B mutant or both together, plated, and placed at 31 C. At intervals duplicate plates were placed at 39 C and left for 4.5 days for plaques to develop. The last column in the table shows that, if kept continuously at 31 C, approximately 80% of infected cells developed into plaques in both the cross and the two controls. If the plates were kept continuously at 39 C practically no plaques developed. Very few plaques arose from the singly infected cells regardless of the time they were raised to 39 C.

TABLE 4. *Recombination frequencies in crosses between R_1d (L_1) and R_2A virus and other mutant classes of reovirus*

Mutants	Recombination frequency (% ts^+)[a]						
	R_2A (201)	R_2B (352)	R_2C (447)	R_2D (585)	R_2E (320)	R_2F (556)	R_2G (453)
R_1d (L_1)	0.05	0.06	0.02	0.03	0.02	0.05	0.04
R_2A (201)		4.5	3.2	6.1	2.6	4.1	3.3

[a] The MOI for each mutant was 10 PFU/cell or 200 particles/cell of R_1d (L_1). Each result given in the table is the average of three to five independent crosses.

TABLE 5. *Determination of recombination by the infectious center test on mixedly infected cells*

Infection[a]	Plated cells (%) giving infectious centers[b]					
	Hours at 31 C, then placed at 39 C				Continuously at 39 C	Continuously at 31 C
	6	12	22	30		
R_2A (201) × R_2B (352)	1	4	67	80	2	84
R_2A (201)	0	1	1	1	0	79
R_2B (352)	0	1	0	1	0	80

[a] MOI, 10 PFU/cell for each mutant.
[b] Values are the averages for duplicate platings of infected cells.

However, for the doubly infected cells maintained for 30 h at 31 C as many plaques developed after the shift to 39 C as in the cells maintained continuously at 31 C. Twenty-four plaques were picked from the plates that had been shifted to 39 C at 30 h, and all were found to be ts^+. These results are interpreted to mean that recombination to ts^+ occurs in the doubly infected cells during replication of the two mutants at 31 C. On shifting the plates to 39 C multiplication of the *ts* mutants ceases, and the ts^+ recombinants form plaques. Although recombination frequencies cannot be determined from this type of experiment it is clear that recombination occurred in all doubly infected cells. Since there were virtually no plaques formed from the cells infected with only one of the mutants, this infectious center assay is a very sensitive test for detecting recombination between *ts* mutants.

The test was applied to crosses between a number of mutants, and the results are shown in Table 6. For each cross the actual number of plaques on each of the plates has been entered to show the variation in the method. The results are consistent with previous work (2, 3) and very clear-cut: both A and D mutants recombine with all other known classes of mutant. On the other hand neither of the two D mutants nor the A mutant show any recombination with the defective virions, R_1d (L_1).

Cross-reactivation (marker rescue) as a means of detecting recombination. We have been carrying out an extensive series of experiments with UV-irradiated reovirus (A. Hossain, unpublished data), and it has been found that both multiplicity reactivation and marker rescue occur in this system with high frequency. These results will be reported in detail elsewhere, but they suggested a marker-rescue type of experiment between defective virions

TABLE 6. *Determination of recombination between ts mutants by infectious center assay*

Infection	No. of plated cells giving infectious centers		
	39 C	31 C	30 h at 31 C, then at 39 C
R_2A (201) × R_2B (352)	2, 5	92, 105	85, 97
R_2A (201) × R_2C (447)	1, 3	80, 83	77, 86
R_2A (201) × R_2D (357)	2, 4	88, 95	93, 100
R_2A (201) × R_2D (585)	2, 3	73, 91	71, 82
R_2A (201) × R_2E (320)	0, 1	62, 93	74, 85
R_2A (201) × R_2F (556)	3, 5	75, 90	72, 83
R_2A (201) × R_2G (453)	1, 3	81, 84	78, 92
R_2D (357) × R_2B (352)	2, 4	69, 93	70, 91
R_2D (357) × R_2C (447)	2, 3	81, 85	68, 73
R_2D (357) × R_2E (320)	0, 0	78, 87	69, 86
R_2D (357) × R_2F (556)	2, 4	74, 91	81, 101
R_2D (357) × R_2G (453)	1, 3	76, 93	67, 94
R_2D (357) × R_2D (585)	1, 2	68, 81	2, 4
R_2D (357) × R_1d (L_1)	2, 3	72, 77	3, 3
R_2D (585) × R_1d (L_1)	1, 3	65, 73	2, 3
R_2A (201) × R_1d (L_1)	2, 3	82, 93	1, 4
R_2A (201)	0, 0	91, 94	0, 1
R_2B (352)	0, 0	82, 89	0, 2
R_2C (447)	0, 0	76, 90	0, 0
R_2D (357)	0, 0	63, 87	0, 3
R_2D (585)	0, 0	72, 93	0, 2
R_2E (320)	0, 0	74, 102	0, 3
R_2F (556)	0, 0	69, 85	0, 2
R_2G (453)	0, 0	68, 84	0, 1
R_1d (L_1)	0, 0	0, 1	0, 0

and a *ts* mutant in an attempt to demonstrate recombination with the defectives. The results of such an experiment are shown in Table 7, and the details are given in the legend to the table.

Essentially, the experiment involved the UV irradiation of R_1d (L_1) virions, coinfection of cells with the irradiated virions and the A mutant, and plating these cells in an infectious center assay to determine whether ts^+ recombinants occurred as a result of reactivation of the A mutation. As a control wild-type virus R_2 was irradiated and crossed with R_2A virus. First, the irradiated R_2 virus was plated alone at the required MOI in the infectious center assay. Many more plaques were found than expected, indicating that multiplicity reactivation had occurred in a fraction of the cells that received more than one irradiated virus (row i, Table 7). Second, R_2A virus plated in the infectious center assay also gave more plaques than expected (row ii, Table 7). The additional plaques found here probably represent leakiness of the mutant under the conditions used; i.e., the cells had been infected at a high MOI and permitted to remain at 31 C for 30 h before being raised to the nonpermissive temperature. In row iii is shown the result of the cross between UV-R_2 and R_2A. The number of infectious centers found in this cross exceeds by a factor of 4.6 the sum of the plaques obtained in the two preceding independent tests. These excess plaques are ts^+ recombinants formed through rescue of the A marker by UV-R_2 virus. On the other hand the cross between UV-R_1d (L_1) and R_2A (row v) when compared with R_2A alone (row ii) and UV-R_1d (L_1) alone (row iv) showed no rescue of the A marker. This is further evidence that recombination does not occur between the defective virions and the A mutant.

Kinetics of recombination. The results of a previous section (Table 5) suggested that ts^+ recombinants were present in practically all mixedly infected cells by 22 h after infection. An experiment was done to find the earliest time such recombinants occurred. Cells were harvested at different times after a mixed infection with A and B mutants, lysed artificially, and examined for the frequency of recombination (Fig. 1). At the earliest time progeny virus could be detected in the infected cells the frequency of ts^+ recombinants was approximately 4.5%. Despite a further nearly 1,000-fold increase in yield of virus the recombination frequency remained virtually constant. This result is similar to one reported by Fields (2) but is more definitive at the early period of the infectious cycle.

TABLE 7. *Cross-reactivation (marker rescue) of R_2A (201) by UV-irradiated R_2 or R_2d (L_1) viruses*

Infection[a]	Infectious centers/10,000 cells plated		
	Found	Expected	Found/ expected
(i) UV-R_2	192	18[b]	11
(ii) R_2A (201)	71	23[c]	3
(iii) UV-R_2 × R_2A (201)	1,213	263[d]	4.6
(iv) UV-R_1d (L_1)	3	0	
(v) UV-R_1d (L_1) × R_2A (201)	77	74[e]	1

[a] R_2 virus was UV-irradiated to 10^{-2} survival (4.6 hits) and R_1d (L_1) received a similar dose. Monolayers of cells in 60-mm plastic dishes were infected with the following combinations of viruses: (i) UV-irradiated R_2, equivalent of 0.2 PFU/cell; (ii) R_2A (201), 10 PFU/cell, titer determined at 31 C; (iii) UV-R_2, equivalent of 0.2 PFU/cell × R_2A (201), 10 PFU/cell; (iv) UV-irradiated R_1d (L_1), 200 particles/cell; and (v) UV-R_1d (L_1), 200 particles cell × R_2A, 10 PFU/cell. Adsorption was for 1 h at room temperature. The cells were then suspended with trypsin, and varying numbers were plated in the infectious center assay. Incubation was for 20 h at 31 C, and the plates were then raised to 39 C for 4 days when the plaques were scored.

[b] Calculated from the number of cells receiving one or more virus particles at an MOI of 0.2 PFU/cell × the UV survival, i.e., $10,000 \times 0.18 \times 10^{-2} = 18$ infectious centers/10,000 cells.

[c] Calculated from the MOI of R_2A and its known efficiency of plating, 39 C/31 C (14, Table 1), i.e., $10,000 \times 10 \times 2.3 \times 10^{-4} = 23$ infectious centers/10,000 cells.

[d] Sum of the infectious centers found, row (i) + row (ii).

[e] Sum of the infectious centers found, row (ii) + row (iv).

DISCUSSION

Defective reovirus lacking the L_1 segment of the genome multiplies in L cells through the helping effect of infectious virions growing simultaneously in the same cell. The helper virus may be wild-type virus of either the type 1 or 3 serotypes or a *ts* mutant growing even at the nonpermissive temperature (8, 13, 14). There appears to be very little mutual interference between the viruses in coinfections at the permissive temperature, since both infectious and defective viruses multiply to relatively high yields (13, 14). Recombination occurs between the various classes of *ts* mutants to a level of 3 to 8% (2, 3) (Table 1). In view of these various characteristics one would expect to find recombination between the defective virions and all

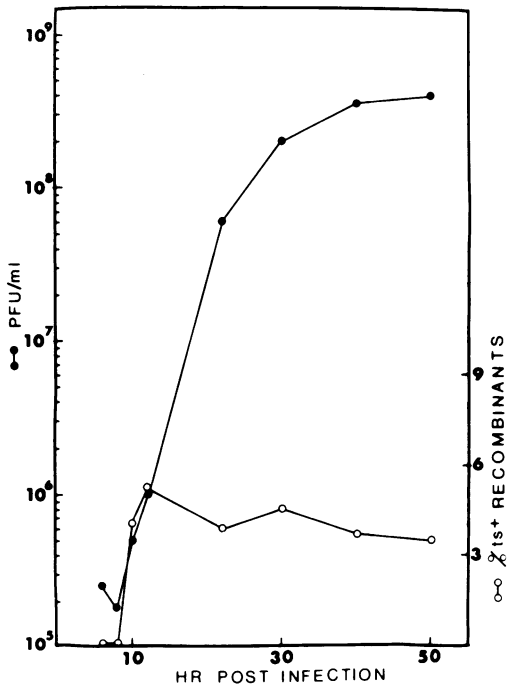

FIG. 1. *Viral growth curve and kinetics of recombination in a mixed infection with R_2A (201) and R_2B (352) mutants at 31 C. MOI was 10 PFU/cell for each mutant.*

the classes of *ts* mutants except that bearing the mutation in the L_1 segment. We have already provided evidence that the class C mutation is in the L_1 segment (13).

Contrary to expectation there was no detectable recombination in the standard tests between defective virions and any known class of *ts* mutants (Table 1). This conclusion is supported by the results of the very sensitive infectious center assay for recombination. In this series of experiments no recombination was detected between defective virus and two D class and one A class mutants, although in crosses between different classes of *ts* mutants virtually all coinfected cells gave rise to ts^+ recombinants. Moreover, the marker rescue assay, a somewhat different type of test, again showed no recombination between defective virus and the class A mutant. Although the infectious center and marker rescue assays were not applied to all class of *ts* mutants, the results that were obtained serve to reinforce the conclusions already drawn from the standard tests. It might be pointed out in passing that the infectious center assay as applied in the present work provides a rather simple and sensitive method for classifying newly isolated *ts* mutants of reovirus into the various groups.

There is no ready explanation for the lack of recombination between defective reovirions and the different classes of *ts* mutants. The deleted L_1 segment bears the class C *ts* mutation, codes for a *trans* function (14), and is involved in replication of the viral genome (15). While the replicative function of the L_1 segment can be readily provided by a helper virus, apparently the capacity for recombination cannot and R_1d (L_1) virions are rec^- mutants. This suggests that the physical presence of the L_1 segment is essential for recombination to occur between reovirus mutants and implies that there is a specific arrangement, and perhaps physical linkage, of the ten genomic segments. A test of this idea would be possible if other deletion mutants were available to cross with the *ts* mutants, and we are presently attempting to isolate pure populations of such defectives. It should be mentioned that lack of recombination between reovirus defectives and *ts* mutants is not unique to the reovirus system. The "von Magnus particles" of influenza virus bear a large deletion of the genome (8), and these particles apparently do not recombine with *ts* mutants of the virus (5). Probably another example of this phenomenon is the lack of recombination between the defective Bryan strain of Rous sarcoma virus and its helper leukosis virus (6). Reovirus, influenza virus, and Rous sarcoma virus all have segmented genomes, and the fact that certain defectives cannot recombine in all three systems suggests that recombination with these viruses might be a good deal more complex than a simple reassortment of the segments during their multiplication.

Some further insight into the mechanism of recombination in reovirus can be gained from the observation (Fig. 1) that the frequency of recombination does not increase during viral replication. It has been established that replication of the viral genome is an asymmetric process (11). That is, single-stranded RNA (ssRNA) transcripts of the parental viral genome synthesized by the virion polymerase (1, 12) are utilized as templates for the synthesis of the complementary strand, and thus the dsRNA progeny genome is formed. The evidence is strong that dsRNA is never free in the cell (4) and that it is synthesized in association with viral proteins in partially formed subviral structures which then go on to become mature virions (10, 17, 18). In this context the constant frequency of recombination during the infectious cycle suggests that progeny genomes are synthesized exclusively with transcripts from parental virions. This means that while progeny genomes are certainly transcribed (16) the

transcripts are used only as mRNA and not as precursors of dsRNA. Furthermore, recombination most likely occurs at the ssRNA level. Since it is difficult to visualize how completed dsRNA segments could reassort between two genomes when synthesis of each genome is being carried out in its individual viral structure, we suggest that, in fact, the reassortment may take place between single-stranded transcripts prior to their association with the replicating structures. This postulate would satisfy the requirements of the recombination results of Fig. 1 but biochemical evidence bearing on the mechanism may not be easy to obtain.

ACKNOWLEDGMENTS

This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada.

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